

Critical Points of Tumor Necrosis Factor Action in Central Nervous System Autoimmune Inflammation Defined by Gene Targeting

By Heinrich Körner,* D. Sean Riminton,* Deborah H. Strickland,*
Frances A. Lemckert,* John D. Pollard,‡ and Jonathon D. Sedgwick*

*From the *Centenary Institute of Cancer Medicine and Cell Biology, Royal Prince Alfred Hospital, Sydney, New South Wales, 2050 Australia; and the †Department of Medicine (Neurology), University of Sydney, Sydney, New South Wales, Australia*

Summary

Tumor necrosis factor (TNF)-dependent sites of action in the generation of autoimmune inflammation have been defined by targeted disruption of TNF in the C57BL/6 mouse strain. C57BL/6 mice are susceptible to an inflammatory, demyelinating form of experimental autoimmune encephalomyelitis (EAE) induced by the 35–55 peptide of myelin oligodendrocyte glycoprotein. Direct targeting of a strain in which EAE was inducible was necessary, as the location of the TNF gene renders segregation of the mutated allele from the original major histocompatibility complex by backcrossing virtually impossible. In this way a single gene effect was studied. We show here that TNF is obligatory for normal initiation of the neurological deficit, as demonstrated by a significant (6 d) delay in disease in its absence relative to wild-type (WT) mice. During this delay, comparable numbers of leukocytes were isolated from the perfused central nervous system (CNS) of WT and TNF^{-/-} mice. However, in the TNF^{-/-} mice, immunohistological analysis of CNS tissue indicated that leukocytes failed to form the typical mature perivascular cuffs observed in WT mice at this same time point. Severe EAE, including paralysis and widespread CNS perivascular inflammation, eventually developed without TNF. TNF^{-/-} and WT mice recovered from the acute illness at the same time, such that the overall disease course in TNF^{-/-} mice was only 60% of the course in control mice. Primary demyelination occurred in both WT and TNF^{-/-} mice, although it was of variable magnitude. These results are consistent with the TNF dependence of processes controlling initial leukocyte movement within the CNS. Nevertheless, potent alternative mechanisms exist to mediate all other phases of EAE.

Studies in vivo point to the importance of TNF in the pathogenesis of autoimmune inflammation (for review see reference 1). Experimental autoimmune encephalomyelitis (EAE), a central nervous system (CNS) autoimmune inflammatory disease, is particularly well studied in this context. EAE follows the recognition of myelin antigen in the CNS by specific autoreactive TH1 CD4⁺ T cells (2, 3). This recognition leads to T cell and macrophage infiltration of the CNS; cytokine secretion, including TNF, lymphotoxin (LT)- α , and IFN- γ (4); loss of blood brain barrier integrity; and, in some cases, antigen-specific tissue damage in the form of demyelination (2).

Inhibitors of TNF consistently prevent or attenuate the clinical course of EAE (5–8). The mechanism of inhibition remains undefined, as TNF has the potential to contribute

to CNS injury at many levels, including via effects on cell adhesion (3), by macrophage activation (9), and by direct cytolysis of oligodendrocytes, the myelinating cell of the CNS (10). The complex interactions between TNF, its homologue LT- α , and their receptors (11), have prevented the precise definition of the critical points of action for TNF in any stage of CNS autoimmune inflammation, or indeed in any inflammatory process.

Mice lacking TNF and/or LT- α can now be applied to this problem. In a recent report, 129 strain mice lacking both TNF and LT- α , and interbred with C57BL/6-strain mice or crossed to the EAE-susceptible SJL mouse strain (12), were shown to be susceptible to a CNS inflammatory disease after immunization with a number of different myelin antigens. Some autoantigen combinations induced a rapidly lethal atypical form of disease. The authors concluded that neither TNF nor LT- α was required for EAE induction. Some properties of the mice used in Frei et al.

H. Körner, D.S. Riminton, and D.H. Strickland contributed equally to this study.

(12) study render an interpretation of the experimental outcome difficult. First, these mice lack all lymph nodes as a result of the absence of LT- α (13). Second, the TNF/LT gene loci reside within the MHC, a region strongly linked to autoimmune disease susceptibility (14) and comprising not only genes encoding for MHC class I and II and the TNF and LT molecules, but also complement components and molecules involved in antigen processing. Thus, attempts to backcross the TNF/LT mutations onto another strain will create individually variable congenic segments, the majority of which will maintain the MHC locus profile linked to the mutated allele. For this reason, the use of a directly targeted, disease-susceptible mouse strain represents a major theoretical advantage.

To explore in detail the role of TNF in autoimmune inflammation in the CNS, TNF gene-deleted C57BL/6 mice normally susceptible to EAE induced by the 35–55 peptide of myelin oligodendrocyte glycoprotein (MOG) were generated (15). Analysis of these mice points to a particular dependence on TNF for the early inflammatory phase of EAE, and specifically to the processes involved in the formation of perivascular cuffs within the CNS. The existence of potent alternative pathways of CNS inflammation and demyelination is demonstrated.

Materials and Methods

Generation of C57BL/6-strain TNF^{-/-} Mice. Construct design, use of the BL/6 III C57BL/6 embryonic stem cells, and generation and characterization of C57BL/6-strain TNF^{-/-} mice have been previously described (15). TNF^{-/-} specific pathogen free mice were maintained as a homozygous colony in the Centenary Institute animal facility (Sydney, Australia). Control wild-type (WT) C57BL/6J-strain mice were bred in-house or obtained from CULAS Ltd. (Sydney, Australia).

Induction of EAE. EAE was actively induced in adult (8–12-wk-old) TNF^{-/-} and WT C57BL/6 mice by subcutaneous tail-base injection of 50 μ g MOG peptide (35-MEVGWYRSPFSR-VVHLYRNGK-55; reference 16) in CFA containing 1 mg of heat-inactivated H37RA *Mycobacterium tuberculosis* (DIFCO Laboratories, Inc., Detroit, MI). 200 ng of pertussis toxin (LIST Biol. Labs., Inc., Campbell, CA), was injected intravenously on days 0 and 2. Neurological deficits were quantified according to an arbitrary clinical scale: 0, normal; 1, flaccid tail; 2, hind limb weakness or abnormal gait; 3, severe hind limb weakness, with loss of ability to right from supine; 4, hind quarter paralysis; 5, forelimb weakness, moribund; 6, death. All animal procedures were approved by the Animal Care and Ethics Committee of the University of Sydney (Sydney, Australia).

Assessment of T Cell Activation and Antibody Production. Groups of WT and TNF^{-/-} mice were immunized (day 0) with MOG peptide/CFA as above, but without the pertussis toxin injection. For assessment of T cell proliferation and IFN- γ production, draining lymph node cells were harvested on days 9 and 5, respectively, after MOG peptide/CFA. Proliferation was assessed using 2×10^5 viable cells/well in the presence of MOG peptide (10 μ g/ml final concentration) or control antigens. T cell proliferative responses were quantified at 96 h after a 14-h pulse with [³H]thymidine. Supernatants for IFN- γ determinations were generated by culture of cells at $0.5\text{--}1 \times 10^7$ /ml with MOG peptide

(20 μ g/ml), or no antigen, for 60 h. IFN- γ was quantitated by sandwich ELISA. For examination of humoral responses, groups of MOG peptide/CFA-challenged mice were secondarily immunized with MOG/IFA on day 9 and were bled for serum collection on day 14. MOG peptide-specific IgG responses were evaluated by ELISA using plates coated with avidin and biotinylated peptide and by detection of antibody with alkaline-phosphatase-conjugated sheep anti-mouse IgG (Sigma Chemical Co., St. Louis, MO).

Flow Cytometry. Flow cytometric analysis of CNS-associated leukocytes was performed on collagenase-digested tissue after heparin-saline perfusion of animals. Isolation and purification steps were adapted from a previously described method (17). Antibodies used in flow cytometry were FITC-conjugated hamster anti-murine α/β -TCR (H57-597), PE-conjugated rat anti-CD45 (30F11.1; PharMingen, San Diego, CA) and relevant isotype control reagents (to set compensation levels and analysis gates). Flow cytometry was performed on a FACStar^{plus}™ using CellQuest analysis software (Becton Dickinson, San Jose, CA).

Immunohistology and Neuropathology. Tissue specimens from various regions of the CNS of nonperfused mice were embedded in Tissue Tek OCT compound (Sakura Finetek, Torrance, CA) and 6- μ m serial cryostat sections were prepared. Sections were stained via the immunoperoxidase technique using HRP-conjugated rabbit anti-rat Ig (DAKO, Carpinteria, CA), and counterstained with hematoxylin. Rat mAb reactive with mouse cell-surface antigens were: GK1.5 (CD4); M1.70 (CD11b/Mac-1); YBM142.2.2 (CD45, supplied by Dr. S. Cobbold, Oxford University, Oxford, UK); 429 (vascular cellular adhesion molecule [VCAM]-1; PharMingen); and isotype control reagents R-35-95 (rat IgG2a, PharMingen) and YKIX16.13 (rat IgG2b, supplied by Dr. S. Cobbold). Neuropathological assessment for demyelination was performed on CNS tissue obtained from animals perfused with warm PBS, followed by 4% paraformaldehyde and 2.5% glutaraldehyde in PBS. Tissue was post-fixed in Dalton's chrome osmium solution and dehydrated in graded concentrations of ethanol and acetone. Transverse sections of spinal cord (0.25 μ m) were stained with toluidine blue. All sections were examined and photographed using standard bright-field optics.

Results

TNF^{-/-} Mice Exhibit Normal MOG Peptide-specific Cell-mediated and Humoral Immune Responses. A combination of cell-mediated and humoral immune responses is considered important for the full manifestation of EAE, particularly in relation to demyelination, in which a clear role for antibody to myelin components, including MOG (18), has been demonstrated. To determine whether mice lacking TNF were deficient in their immune responsiveness to MOG peptide, mice were immunized and analyzed for MOG peptide-specific T cell proliferation, IFN- γ production, and IgG production, as detailed in Materials and Methods. No significant differences in any of these parameters were observed between TNF^{-/-} and WT mice (data not shown).

Onset of EAE in TNF^{-/-} C57BL/6 Mice Is Substantially Delayed. Upon challenge with the encephalitogenic MOG 35–55 peptide, WT mice exhibited signs of clinical disease from day 10 (Fig. 1 A), manifest as symmetrical ascending motor deficits. Disease severity then increased rapidly to

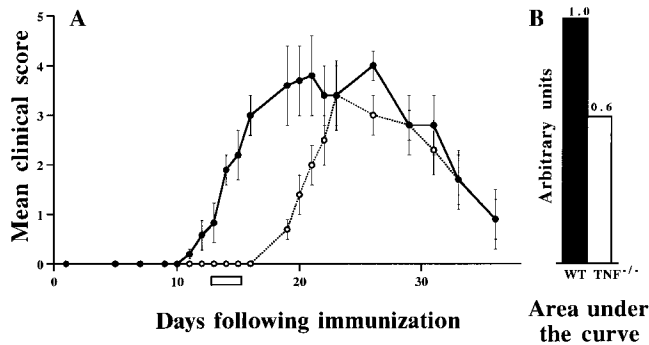


Figure 1. Natural history of EAE in WT and TNF^{-/-} C57BL/6 mice. (A) Mean clinical EAE scores (± SEM) of WT mice (●, *n* = 6) and TNF^{-/-} mice (○, *n* = 8) after immunization with MOG/CFA. The horizontal bar at days 13–15 indicates a time point of closer examination, referred to in Fig. 2 and in the text. (B) As a measure of the relative susceptibility of WT and TNF^{-/-} mice to neurological deficits induced by immunization with MOG, areas under the curves in A were determined. Results are representative of four separate time course studies.

reach a peak at day 20, followed by gradual recovery over the next 20 d to a relatively mild deficit that persisted for the life of the mouse (data not shown). Although TNF^{-/-} mice did develop EAE after MOG peptide challenge, the onset of clinical disease was substantially and reproducibly delayed (Fig. 1 A). The rate of progression once disease became established and the eventual peak severity of disease in TNF^{-/-} mice were comparable to WT mice. Both WT and TNF^{-/-} mice recovered simultaneously despite the initial delay in disease onset in mice lacking TNF. Thus, the overall disease course was reduced to 60% of the course in control mice in the absence of TNF (Fig. 1 B). Like WT mice, mice lacking TNF maintained a mild level of disability for an extended time after resolution of the initial peak clinical deficit.

The clear conclusion that can be drawn from these results is that TNF is required for the normal initiation of the neurological deficit in EAE, but is not a necessary factor for disease progression or for recovery from the acute clinical illness.

Leukocytes Accumulate in the CNS in the Absence of TNF but Formation of Perivascular Cuffs is Delayed. Since TNF appeared to be an essential participant in the early events leading to the development of EAE, a series of experiments was performed to establish how the absence of TNF affected the CNS inflammatory process, using the time point at which the discrepancy between the clinical scores of TNF^{-/-} and WT mice was maximal (Fig. 1 A, days 13–15, horizontal bar). Immunohistological analysis of mice at this time revealed small but frequently detectable accumulations of leukocytes (CD45⁺) in TNF^{-/-} mice (Fig. 2 A, upper panels), but a marked reduction in discrete perivascular cuffs of leukocytes relative to WT. On the other hand, no detectable accumulations of leukocytes (CD45⁺) were found in the CNS of normal nonimmunized mice (data not shown). Clear evidence for the existence of immunological activation within the CNS was revealed by staining for the adhe-

sion molecule VCAM-1 (Fig. 2 A, lower panels), which was substantially upregulated on vascular endothelium throughout the CNS of TNF^{-/-} mice.

When this observation was extended to an investigation of the immune response throughout the entire CNS, the kinetics of total inflammatory cell accumulation in the CNS were found to be remarkably similar in WT and TNF^{-/-} mice (Fig. 2, B and C). Cells isolated from PBS-perfused CNS tissue of WT and TNF^{-/-} mice were quantified at intervals after immunization (Fig. 2 B) and phenotyped by flow cytometry using criteria previously developed (17). Flow-cytometric analysis at day 15 (Fig. 2 C) revealed the presence of equivalent numbers of α/β T cells (population 1, CD45^{hi}α/βTCR⁺), non-T inflammatory cells (population 2, CD45^{hi}α/βTCR⁻), the majority of which were macrophages (data not shown), and microglia (population 3, CD45^{low}α/βTCR⁻). Relative to other populations isolated (T cells, and microglia), there were more inflammatory macrophages in the CNS of WT than TNF^{-/-} mice (Fig. 2 C, population 2). A small number of T cells and macrophages were isolated from the CNS of nonimmunized WT mice (Fig. 2 C, upper panel), as expected, although resident microglial cells (population 3, CD45^{low}α/βTCR⁻) were readily detectable (17).

These studies support the view that MOG peptide-reactive T cells are generated normally in TNF^{-/-} mice, migrating to and distributing throughout the CNS vasculature and leading to endothelial activation, as evidenced by VCAM-1 upregulation—all outcomes that are not dependent on TNF. However, in the absence of TNF, formation of discrete mature perivascular cuffs of inflammatory cells is significantly retarded.

Peak Disease Inflammation and Demyelination in WT and TNF^{-/-} Mice. At the peak of the disease there was extensive inflammation in the CNS of both WT and TNF^{-/-} mice (Fig. 3 A), characterized by perivascular and submeningeal infiltrates of CD45⁺ cells and microglial activation (Fig. 3 A, arrows). Serial section staining revealed a predominance of macrophages and CD4⁺ T cells (data not shown). A general feature of the immunopathology in TNF^{-/-} mice was a more limited expansion of cells from the perivascular cuff into the parenchyma. Primary demyelination, involving loss of myelin from otherwise viable axons, is a hallmark of the human disease multiple sclerosis, for which EAE serves as an experimental model (2). Primary demyelination was a relatively late event, detected in WT and TNF^{-/-} mice from ~30 d after MOG peptide/CFA immunization and most clearly apparent after the bulk of inflammatory cells had dissipated (Fig. 3 B, day 35). Naked axons of otherwise normal appearance were seen, consistent with the specificity of the immune insult (Fig. 3 B, arrows). A degree of variability in magnitude of demyelination was observed in TNF^{-/-} mice with from one of five mice examined exhibiting few if any demyelinated axons (data not shown), to the one mouse illustrated here (Fig. 3 B, day 40) with a level of demyelination indistinguishable from WT mice. A more extensive comparison of WT and TNF^{-/-} mice is currently underway to determine whether

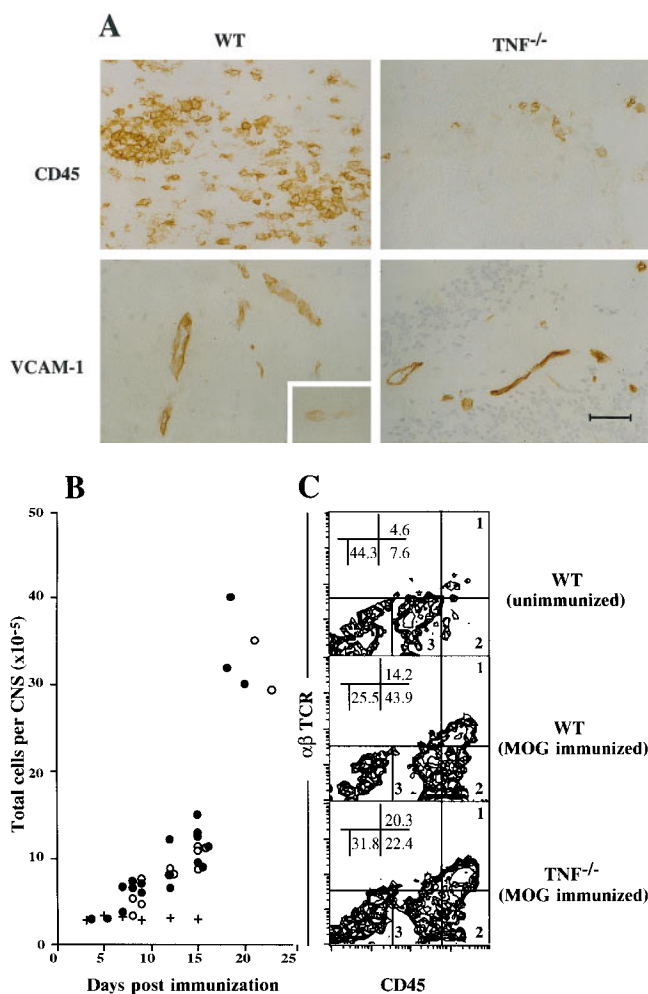


Figure 2. Characterization of the early CNS inflammatory infiltrate in WT and TNF^{-/-} mice. (A) Leukocyte inflammation (CD45⁺) and VCAM-1 expression at day 15 (Fig. 1 A, horizontal bar). Tissue sections were derived from brain stem and cerebellum and are representative of tissues throughout the CNS of several WT and TNF^{-/-} mice at this time point. (Inset) VCAM-1 expression of unimmunized C57BL/6J-strain CNS. Bar = 60 μm. (B) Total cell recoveries from the perfused CNS of normal and immunized mice over the course of disease. Replicates concentrated on the early disease phase when TNF^{-/-} mice were not showing signs of clinical disease. For simplicity, individual mice from days 13–15 are all shown at the day 15 time point. Each data point represents a single mouse. +, WT unimmunized. ●, WT MOG immunized. ○, TNF^{-/-} MOG immunized. (C) T cell accumulation in the CNS of normal and MOG-immunized WT and TNF^{-/-} mice at day 15. Pooled cells obtained from two mice in each case were stained and analyzed by flow cytometry. Percentage of total cells in each preparation as defined by populations 1, 2, and 3 (see text) are shown in the inset. CD45⁻ TCR⁻ cells are undefined but would represent nonhematopoietically derived cells including neurons, endothelial cells, astrocytes, and oligodendrocytes.

the levels of demyelination in mice lacking TNF are reduced overall. Nevertheless, TNF is not an obligatory mediator in the demyelinating process.

Discussion

From this study of the course of EAE, clear conclusions can be derived regarding the critical roles for TNF. Of the

three stages of the inflammatory process: initiation, tissue injury, and recovery, TNF appears to play a unique role only in the first. Unexpectedly, the altered characteristics of the inflammatory disease process in TNF^{-/-} mice are consistent with the inefficient movement of cells within the CNS, while normal upregulation of VCAM-1 and the identification of recruited cells at the time of disease delay suggest there are no major deficiencies in vascular adhesion in the absence of TNF. While the precise location of leukocytes within the CNS of TNF^{-/-} mice is unknown during the delayed preclinical phase of EAE (Fig. 2 A, upper right panel), the data nevertheless support the view that extravasation of leukocytes, localization to the perivascular space throughout the CNS, and antigen recognition by MOG-reactive T cells at that site (19, 20) have occurred normally. This conclusion is based on the observation of VCAM-1 upregulation as well as accumulation of leukocytes other than T cells in TNF^{-/-} mice during the preclinical phase (Fig. 2, A and C), events almost certainly necessitating CNS antigen recognition by infiltrating T cells.

A variety of TNF-dependent processes may underlie this unusual phenotype. However, a likely explanation is that the delayed movement of leukocytes within the tissue to form perivascular cuffs reflects a general inability of leukocytes to move correctly in the absence of TNF-inducible chemotactic factors, notably chemokines (21). Consistent with this concept is the defect of cell movement manifest in the altered microarchitectural T and B cell arrangements in lymphoid tissues of TNF^{-/-} mice (15, 22). The potential role of secondary mediators in this process is highlighted by a recent description of mice in which deletion of the gene encoding a putative chemokine receptor, *blr1*, resulted in splenic B cell architecture not unlike that seen in TNF^{-/-} mice (23).

An important outcome of these studies is the demonstration of a potent, but TNF-independent, mechanism producing tissue injury in EAE. The results of studies of collagen arthritis in mice administered TNFR-IgG fusion protein or lacking TNFR-1 (24) are strikingly similar to those reported here in TNF^{-/-} mice with EAE. In particular, in TNFR1-deficient mice, arthritis was of a reduced overall severity, but, once established in an individual joint, progressed in a manner similar to WT mice. Therefore, the processes that lead to tissue damage in EAE and collagen arthritis, once it is initiated, and the eventual peak severity of the diseases, are not due to the actions of TNF alone.

Soluble LT-α homotrimer, a predominantly T cell cytokine with some functional similarities to TNF and binding the same receptors as TNF (11), remains a possible mediator of demyelination as well as of the acute EAE phase. A full analysis of the role of LT-α and -β in EAE in gene-targeted C57BL/6 mice prepared in parallel to the TNF^{-/-} mice is ongoing. These studies are cumbersome, requiring the use of irradiation bone marrow chimeras (13) to generate mice which carry lymphoid tissues but are deficient in LT-α. Lymph nodes were lacking in TNF/LT-α double-deficient mice shown recently to be susceptible to autoimmune CNS inflammation (12). Likely alterations to normal

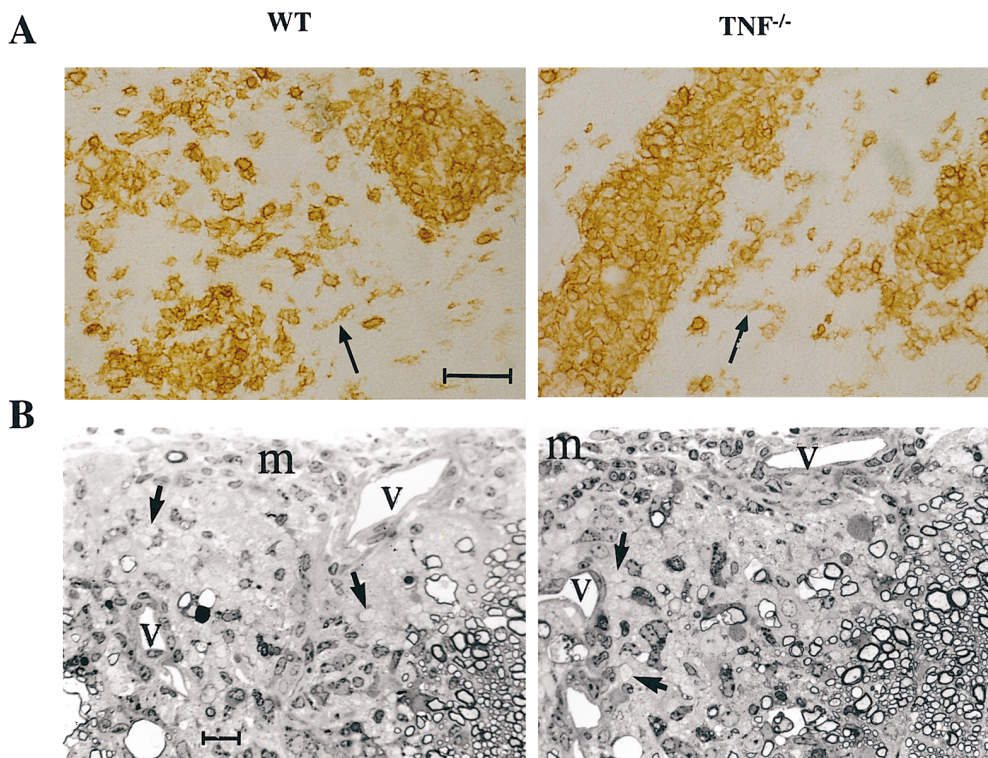


Figure 3. Peak disease inflammation and demyelination. (A) Leukocyte infiltration and formation of perivascular cuffs. Sections were derived from spinal cord of animals harvested at day 19 (WT) and day 23 (TNF^{-/-}) and stained for CD45. CD45⁺ filamentous processes within the CNS parenchyma (arrows) indicate activated microglia. Comparable infiltration was found at all levels of the spinal cord, brain stem, and cerebellum. Bar = 60 μ m. (B) Primary demyelination in WT and TNF^{-/-} mice. Sections were derived from spinal cord of mice harvested at day 35 (WT) and day 40 (TNF^{-/-}). Tissues from WT and TNF^{-/-} mice show a region of comparable perivenous (v) demyelination and gliosis (m) beneath the meningeal surface (m). Arrows indicate naked (demyelinated) axons. In these mice, a similar histological picture was obtained at all levels of the spinal cord. Bar = 12 μ m.

immunological regulation, and the absence of a switched humoral response in these mice (25) have the potential to significantly influence the disease outcome after immunization. As the experiments here have shown, TNF appears to play a critical role in the early inflammatory process in EAE. This same TNF dependency was not revealed in mice lacking both TNF and LT- α (12). Thus, it is difficult to say with certainty at this stage that LT- α plays no role in the EAE disease process in immunologically intact mice.

The requirement for TNF in the normal initiation of autoimmune inflammation, as demonstrated in this study,

may help to explain the therapeutic effectiveness of TNF blocking agents when administered before rather than after disease onset in several disease models (7, 24, 26, 27). Conversely, there is evidence from other models (notably in the rat) that TNF may act as a downstream effector of tissue injury (8, 28, 29), while in this study, once the disease was established, it progressed normally without TNF. Reconciling these apparent discrepancies must await a more detailed understanding of the role of TNF in cell movement within tissues, but also an appreciation of the role that TNF may play in induction of alternative effector pathways.

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Address correspondence to Dr. Jonathon D. Sedgwick, Centenary Institute of Cancer Medicine and Cell Biology, Bldg 93, Royal Prince Alfred Hospital, Missenden Rd., Camperdown, Sydney NSW 2050, Australia. Phone: 61-2-9565-6116; FAX: 61-2-9565-6103; E-mail: j.sedgwick@centenary.usyd.edu.au

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